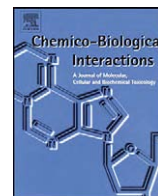


<b>REPORT DOCUMENTATION PAGE</b>			Form Approved OMB No. 0704-0188		
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<b>1. REPORT DATE (DD-MM-YYYY)</b> 2010		<b>2. REPORT TYPE</b> Open Literature		<b>3. DATES COVERED (From - To)</b>	
<b>4. TITLE AND SUBTITLE</b> In vivo reactivation by oximes of inhibited blood, brain and peripheral tissue cholinesterase activity following exposure to nerve agents in guinea pigs			<b>5a. CONTRACT NUMBER</b>		
			<b>5b. GRANT NUMBER</b>		
			<b>5c. PROGRAM ELEMENT NUMBER</b>		
<b>6. AUTHOR(S)</b> Shih, T-M., Skovira, JW, O'Donnell, JC, McDonough, JH			<b>5d. PROJECT NUMBER</b>		
			<b>5e. TASK NUMBER</b>		
			<b>5f. WORK UNIT NUMBER</b>		
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b>  US Army Medical Research Institute of Chemical Defense ATTN: MCMR-CDR-P 3100 Ricketts Point Road  Aberdeen Proving Ground, MD 21010-5400			<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>  USAMRICD-P09-026		
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> US Army Medical Research Institute of Chemical Defense ATTN: MCMR-CDZ-I 3100 Ricketts Point Road  Aberdeen Proving Ground, MD 21010-5400			<b>10. SPONSOR/MONITOR'S ACRONYM(S)</b>		
			<b>11. SPONSOR/MONITOR'S REPORT NUMBER(S)</b>		
<b>12. DISTRIBUTION / AVAILABILITY STATEMENT</b>  Approved for public release; distribution unlimited					
<b>13. SUPPLEMENTARY NOTES</b> Published in Chemico-Biological Interactions ,187, 207-214, 2010 . This research was supported by the Defense Threat Reduction Agency - Joint Service and Technology Office, Medical Science and Technology Division.					
<b>14. ABSTRACT</b> See reprint.					
<b>15. SUBJECT TERMS</b> acetylcholinesterase, cholinesterase reactivation, guinea pig, methoxime, nerve agents, oximes, chemical warfare agents, medical countermeasures					
<b>16. SECURITY CLASSIFICATION OF:</b>			<b>17. LIMITATION OF ABSTRACT</b>  UNLIMITED	<b>18. NUMBER OF PAGES</b>  8	<b>19a. NAME OF RESPONSIBLE PERSON</b> Tsung-Ming Shih
<b>a. REPORT</b> UNCLASSIFIED	<b>b. ABSTRACT</b> UNCLASSIFIED	<b>c. THIS PAGE</b> UNCLASSIFIED			<b>19b. TELEPHONE NUMBER (include area code)</b> 410-436-3414



## *In vivo* reactivation by oximes of inhibited blood, brain and peripheral tissue cholinesterase activity following exposure to nerve agents in guinea pigs<sup>☆</sup>

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### ARTICLE INFO

#### Article history:

Available online 9 March 2010

#### Keywords:

Acetylcholinesterase  
Cholinesterase reactivation  
Guinea pig  
Methoxime  
Nerve agents  
Oximes

### ABSTRACT

This study compared the ability of nine oximes (HI-6, HLö7, MMB-4, TMB-4, carboxime, ICD585, ICD692, ICD3805, and 2-PAM) to reactivate *in vivo* cholinesterase (ChE) in blood, brain, and peripheral tissues in guinea pigs intoxicated by one of four organophosphorus nerve agents. Two bis-pyridinium compounds without an oxime group, SAD128 and ICD4157, served as non-oxime controls. Animals were injected subcutaneously with  $1.0 \times LD_{50}$  of the nerve agents sarin, cyclosarin, VR or VX and treated intramuscularly 5 min later with one of these oximes. Toxic signs and lethality were monitored; tissue ChE activities were determined at 60 min after nerve agent. Some animals exposed to sarin or cyclosarin, with or without non-oxime treatment, died within 60 min; however, no animal treated with an oxime died. For VR or VX, all animals survived the 60 min after exposure, with or without non-oxime or oxime therapy. The four nerve agents caused differential degrees of inhibition in blood, brain regions and peripheral tissues. The tested oximes exhibited differential potency in reactivating nerve agent-inhibited ChE in various peripheral tissues, but did not affect ChE activity in the brain regions. There was no direct relation between blood and peripheral tissues in the reactivating efficacy of oxime treatments. ChE inhibited by sarin was the most susceptible to oxime reactivation while cyclosarin the least susceptible. There was no difference in the ChE reactivating potency between the dimethanesulfonate and dichloride salts of HI-6. MMB-4 significantly reactivated the ChE inhibited by these four nerve agents in blood and all three peripheral tissues of the guinea pig, and among all the oximes tested it was the most effective *in vivo* ChE reactivator against all four nerve agents.

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### 1. Introduction

The ability of oximes to reactivate cholinesterase (ChE) activity inhibited by organophosphorus nerve agents is considered to be the major mechanism of their antidotal action [1]. 2-PAM (pralidoxime) is a mono-pyridinium oxime approved for use in the U.S. for the treatment of nerve agent exposure. Although 2-PAM

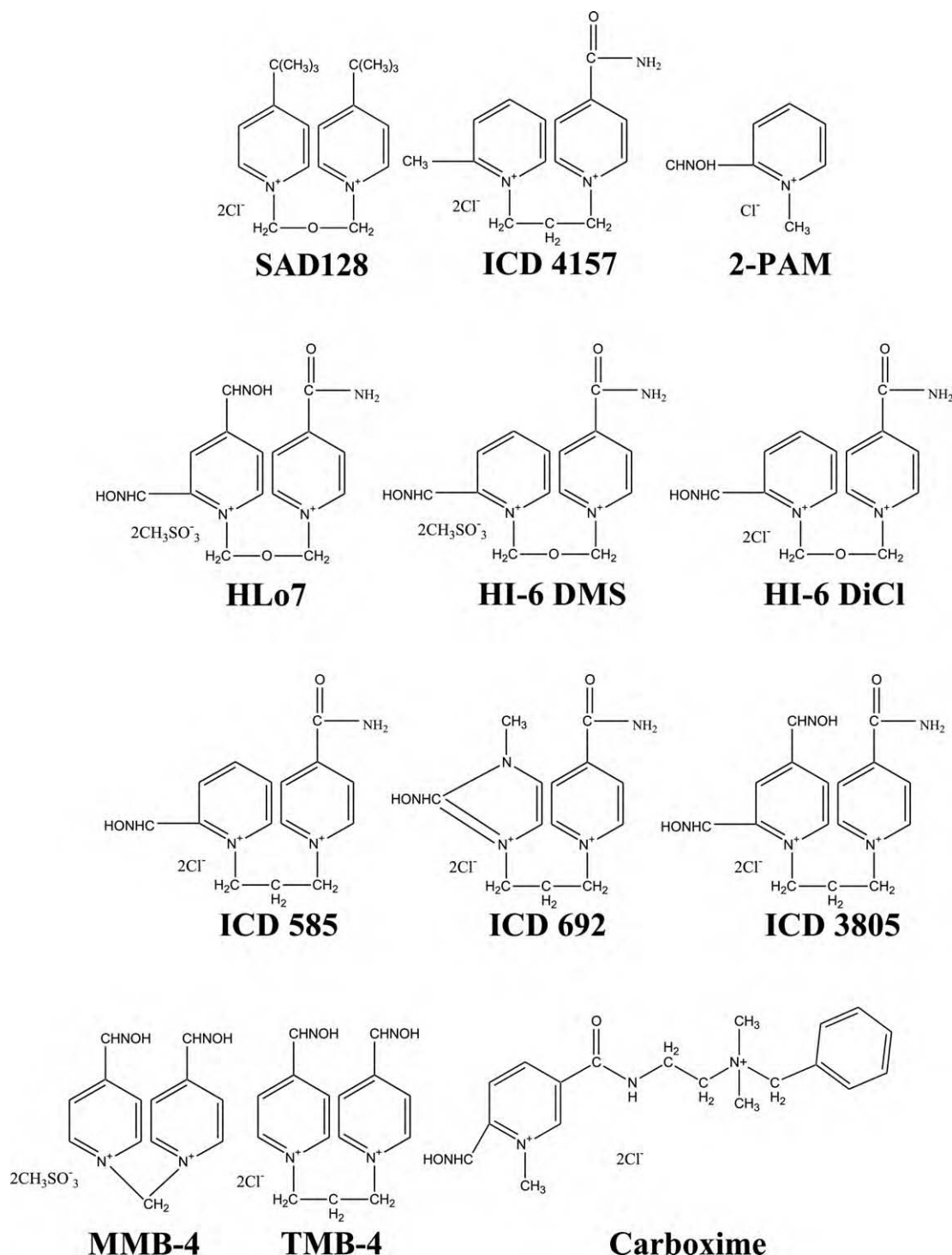
provides adequate protection against some nerve agents, such as sarin (GB) and VX, it is less effective against other nerve agents, such as tabun, soman, cyclosarin (GF), and a Russian V-agent (VR) [2]. For this reason, numerous studies in the last several decades have focused on developing improved oxime reactivators, and several oximes, such as HI-6, HLö7, MMB-4 (methoxime), TMB-4 (trimedoxime), carboxime, ICD585, ICD692, and ICD3805, have been found to possess much better *in vitro* reactivating potency or *in vivo* antidotal capacity against nerve agent intoxication than 2-PAM [3–7]. Although several of these oximes have been studied for their ability to reactivate ChE inhibited by single nerve agents [8–14], their broad-spectrum capacity to reactivate ChE activity inhibited by several nerve agents has not yet been evaluated and compared systematically. The ideal oxime should be able to effectively reactivate ChE inhibited by nerve agents of diverse structures.

The objective of this study was primarily to compare the ability of several oximes (HI-6, HLö7, MMB-4, TMB-4, carboxime, ICD585, ICD692, and ICD3805) with 2-PAM (see Fig. 1) to reactivate blood, brain, and peripheral tissue ChE in guinea pigs intoxicated by four nerve agents. A second objective was to compare the ChE reactivating

<sup>☆</sup> Research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and adhered to principles stated in the *Guide for the Care and Use of Laboratory Animals*, by the Institute of Laboratory Animal Resources, National Research Council. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). The opinions or assertions contained herein are the private views of the authors, and are not to be construed as reflecting the views of the Department of the Army or the Department of Defense.

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**Fig. 1.** Chemical structures of non-oximes (SAD128 and ICD 4157) and oximes (2-PAM, HLo7 [ICD 2445], HI-6 DMS, HI-6 DiCl, ICD 585, ICD 692, ICD 3805, MMB-4 [ICD 039], TMB-4, and carboxime).

vating potency of the two salts of HI-6 under these conditions. The dichloride (DiCl) salt of HI-6 was studied for years [9,11,12], but the dimethanesulfonate (DMS) salt of HI-6 is more water soluble and is proposed to be utilized in a new HI-6 autoinjector in development [15]. Studies with the DMS salt are needed to investigate its pharmacodynamic *in vivo* equivalency with the DiCl salt, as was reported for *in vitro* study [16]. Two structurally similar compounds without an oxime moiety (SAD128 and ICD4157) were included to serve as negative controls in ChE reactivation (Fig. 1). The overall goals were to generate a database for selecting a broad-spectrum

oxime that is significantly more effective than 2-PAM against a spectrum of nerve agents [17,18].

## 2. Materials and methods

### 2.1. Subjects

Male Hartley guinea pigs (Charles River Labs, Kingston, NY; CrI:(HA) BR COBS) weighing 250–300 g served as subjects. They were housed individually in temperature (21 ± 2 °C)- and humid-

ity ( $50 \pm 10\%$ )-controlled quarters and maintained on a 12-h light–dark schedule (with lights on at 0600 h). Laboratory chow and tap water were freely available. Animals were acclimated for 1 week prior to experimentation.

## 2.2. Materials

Saline (U.S.P.), Attane™ (Isoflurane, U.S.P.), and heparin sodium were purchased from Braun Medical Inc. (Irvine, CA), Minrad, Inc. (Bethlehem, PA), and U.S.P., Inc. (Rockville, MD), respectively. 2-PAM was purchased from Ayerst Labs, Inc. (New York, NY). HLö7 (1-[[[4-(aminocarbonyl)pyridinio]methoxy] methyl]-2,4-bis[(hydroxyimino) methyl]pyridinium dimethanesulfonate), MMB-4 (1,1'-methylenebis[4-[(hydroxyimino) methyl] pyridinium] dichloride), HI-6 DiCl (1-(((4-(aminocarbonyl)pyridinio) methoxy)methyl)-2-((hydroxyimino)methyl) pyridinium dichloride), HI-6 DMS, TMB-4 (1,1'-trimethyl bis-[4-formyl pyridinium chloride] dioxime), carboxime (1,4-methyl-5-[2'-(benzyltrimethylammonium)ethyl] carbamoylpyridinium-2-aldoxime dichloride), ICD585 (1-(4-(aminocarbonylpyridinio)-3-(2-hydroxyiminomethylpyridinio)propane dichloride monohydrate), ICD692 (1-[1-(2-hydroxyiminomethyl-3-methyl) imidazo]-3-[4-carbamoylpyridinium]-propane dichloride hydrate), ICD3805 (3-[4-carbamoyl-1-pyridino]-1-[2,4-bis(hydroxyiminomethyl)-1-pyridino]propane dichloride hydrate), ICD4157 (1-[3-[4-(aminocarbonylpyridinio) propyl]-2-methylpyridinium dichloride hydrate), and SAD128 (1,1'-[oxybis(methylene)]bis[(4-tert-butyl)-pyridinium] dichloride) were obtained from the depository at the Division of Experimental Therapeutics, Walter Reed Army Institute of Research (Silver Spring, MD). Sarin (GB; isopropyl methylphosphonofluoridate), cyclosarin (GF; cyclohexyl methylphosphonofluoridate), VX (o-ethyl S-(2-(diisopropylamino)ethyl) methylphosphonothioate), and a Russian V-type agent designated VR (o-isobutyl S-(2-(diethylamino)ethyl)methylphosphonothioate) were obtained from the US Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD). Nerve agents were diluted in ice-cold saline prior to subcutaneous (sc) injection. All non-oxime and oxime compounds were prepared in saline for intramuscular (im) injection. Injection volume was 0.5 ml/kg for the administration of all nerve agents and oxime compounds.

## 2.3. Experimental procedures

One to 3 days prior to the experiment, blood ( $\sim 0.5$  ml) was drawn using a toenail clip method [19] and was collected into a 1.0-ml microfuge tube containing 50  $\mu$ l of heparin sodium (15 U/ml) to determine baseline ChE activity in whole blood (WB) and red blood cell (RBC). On the day of the study, groups of guinea pigs were injected sc with either saline (0.5 ml/kg) or a  $1.0 \times LD_{50}$  dose of GB (42.0  $\mu$ g/kg), GF (57.0  $\mu$ g/kg), VR (11.3  $\mu$ g/kg) or VX (8.0  $\mu$ g/kg). Five minutes later, when the inhibition of ChE activity by these nerve agents reached maximum in the blood [20], saline, SAD128 (20.7 mg/kg), ICD 4157 (20.1 mg/kg), MMB-4 (19.1 mg/kg), carboxime (24.0 mg/kg), HLö7 (30.2 mg/kg), HI-6 DMS (27.8 mg/kg), HI-6 DiCl (21.9 mg/kg), 2-PAM (25.0 mg/kg), ICD 585 (21.8 mg/kg), ICD 692 (22.0 mg/kg), ICD 3805 (23.2 mg/kg) or TMB-4 (20 mg/kg) was given im. A group of animals that received both sc saline (i.e., no nerve agent) and im saline (i.e., no oxime) injections served as overall controls (saline/saline group).

The dose of 2-PAM was equivalent to 145.0  $\mu$ mol/kg, im, which is equivalent to 3 autoinjector doses (as in Mark I Nerve Agent Antidote Kit) in a 70-kg person. The dose of TMB-4 was 43  $\mu$ mol/kg, equivalent to its 1/4  $LD_{50}$  dose. The dose of other oximes studied was 58.0  $\mu$ mol/kg, im, which was equivalent to the maximum 3

autoinjector doses to be given to a 70-kg person (based on HI-6 DiCl) [15]. ICD4157 and SAD128 served as negative controls. ICD4157 is structurally similar to ICD 585 and SAD128 is structurally similar to HI-6. The dose of ICD4157 was equivalent to 58  $\mu$ mol/kg, while the dose of SAD128 used was 53.6  $\mu$ mol/kg, equivalent to its 1/4  $LD_{50}$  dose.

Sixty minutes after sc saline or nerve agent administration, the animals were deeply anesthetized with isoflurane and euthanized by decapitation. Shortly before anesthesia, the severity of toxic signs of each animal was scored. Blood ( $\sim 0.5$  ml) was collected into a 1.0-ml microfuge tube containing 50  $\mu$ l of heparin sodium solution (15 U/ml). For the WB samples, 20  $\mu$ l of blood was diluted 1:25 in 1% Triton-X 100 solution. For the RBC samples, the original blood sample was centrifuged for 5 min at 14,000 rpm, and 10  $\mu$ l of the packed RBC was then diluted 1:50 in 1% Triton-X 100 solution. Brain regions (brainstem, cerebellum, cortex, hippocampus, midbrain, spinal cord [cervic-thoracic region] and striatum) and peripheral tissue (diaphragm [whole], heart, and skeletal muscle [*gastrocnemius*]) were dissected. Brain samples were diluted 1:20, while peripheral samples were diluted 1:5, in 1% Triton-X 100 solution and then homogenized. The homogenates were then centrifuged ( $31,000 \times g$  at  $4^\circ C$ ; 20 min for brain and 30 min for peripheral tissues) and the supernatant was decanted and kept frozen at  $-80^\circ C$  until ChE analysis.

Processed blood, tissue and brain samples were analyzed for ChE activity and protein concentrations according to the methods described by Shih et al. [20,21], based on the colorimetric method of Ellman et al. [22] and a BCA protein assay (Pierce Biotechnology, Inc.). Briefly, in the modified Ellman method, 7  $\mu$ l of sample (or enzyme standard), 20  $\mu$ l of  $H_2O$ , 200  $\mu$ l of DTNB reagent, and 30  $\mu$ l of acetylthiocholine iodide substrate was added to each well of a 96 well microplate (samples run in triplicate), and mean activity was determined by applying Beer–Lambert's Law, incorporating the observed change in absorbance, known extinction coefficient of the DTNB ion, path-length correction, and dilution factor. All sample values were well within the linear range of the standard curve. Protein concentrations of brain and peripheral tissue samples in the BCA assay were determined by interpolating from a standard curve, and applied to ChE activity from the same tissue sample for final values in  $\mu$ mol/(g protein min). Each tissue sample was normalized as a percentage of the average saline/saline control group value for that tissue type. Post-exposure blood enzyme activity ( $\mu$ mol/(ml min)) was normalized as a percentage of each sample's corresponding baseline activity determined 1–3 days prior to study.

## 2.4. Data analysis

Statistical analysis was performed using a one-way ANOVA to compare across tissues for ChE activity, among tissues across nerve agents, and across treatment groups for each nerve agent. A post hoc Tukey test was used for multiple comparisons. Statistical significance was defined as  $p < 0.05$ . Only comparisons between each treatment group in which ChE activity was significantly higher than the activity in the nerve agent/saline-treated group or the 2-PAM-treated group are discussed. In this report, the ChE activity reactivated by the specific oxime in any tissue is considered to be that portion of the activity in a nerve agent/oxime-treated group that significantly exceeded the activity in the nerve agent/saline-treated group (at 60 min).

We divided the analysis of ChE reactivation data into two compartments: the peripheral tissues and the blood components. A “+” sign was assigned for each of the peripheral tissues or blood components, when an oxime treatment significantly reactivated nerve agent-inhibited ChE. For example, if an oxime treatment significantly reactivated ChE in all three peripheral tissues its cell received

“+++” maximum, or in the two components of blood its cell received “++” maximum. When an oxime treatment did not significantly reactivate a nerve agent-inhibited ChE in any tissue, it was assigned a “–” sign.

### 3. Results

#### 3.1. Signs of toxicity and lethality

Under the conditions of this study all guinea pigs exhibited signs of nerve agent intoxication (salivation, rhinorrhea, tremors, muscle fasciculations, convulsions) at 60 min following the  $1.0 \times LD_{50}$  dose of GB, GF, VR or VX. In the case of GB and GF exposure, 3 of 21 (14%) and 20 of 27 (74%) animals, respectively, that were treated with saline died within 60 min. One of 9 animals (11%) exposed to GF and treated with SAD128 died during this period. One of 8 (13%) and 12 of 19 (63%) animals exposed to GB and GF, respectively, and treated with ICD4157 died. No animal died when treated with any oxime. In the case of VR and VX exposure, all animals survived the 60 min after exposure with or without oxime or non-oxime therapy.

#### 3.2. ChE activity in brain regions

The striatum ( $389.3 \pm 9.2 \mu\text{mol}/(\text{g protein min})$ ) has the highest and the cortex ( $56.9 \pm 1.4 \mu\text{mol}/(\text{g protein min})$ ) has the lowest basal ChE activity among all brain regions. The rank order of basal ChE activity from high to low was striatum > cerebellum > brainstem > spinal cord > midbrain > hippocampus > cortex. Nerve agent-inhibited ChE activities in any of the brain regions were not affected by the oxime treatments, with two exceptions. In the cortex, treatment with HLö7 significantly elevated the ChE activity inhibited by VR from  $19.4 \pm 1.8\%$  (mean  $\pm$  SEM) to  $26.6 \pm 2.5\%$  of control. In the spinal cord, treatment with carboxime significantly elevated the ChE activity inhibited by VX from  $46.4 \pm 3.2\%$  to  $55.2 \pm 2.4\%$  of control. The non-oximes (SAD128 and ICD4157) did not affect or modify the ChE activities inhibited by any nerve agent in any brain region.

#### 3.3. ChE activity in peripheral tissues (Table 1)

Basal ChE activities in the diaphragm, heart and skeletal muscle were  $14.3 \pm 0.8$ ,  $18.2 \pm 0.6$ , and  $10.5 \pm 0.4 \mu\text{mol}/(\text{g protein min})$ , respectively. There was no significant difference among them. Table 1 summarized the ChE activity in peripheral tissues and blood components following exposure to nerve agents and saline or 6 top oxime treatments. GB and VX produced in the diaphragm the same degree of ChE inhibition (72–74%), which was significantly higher than that induced by GF (65%) or VR (52%). Similarly, in the skeletal muscle, GB and VX produced ChE inhibition (66–68%) that was significantly greater than was produced by GF (52%) or by VR (39%). In the heart, the four nerve agents produced ChE inhibition that was significantly different among them. The rank order of ChE inhibition from high to low was GB > VX > GF > VR.

##### 3.3.1. Diaphragm tissue

All oximes studied were capable of significantly reactivating diaphragm ChE activity inhibited by GB. MMB-4 and HLö7 showed the highest levels of reactivation, returning ChE activity from  $25.7 \pm 2.2\%$  to  $90.1 \pm 2.4\%$  and  $80.1 \pm 1.4\%$  of the control, respectively. They reactivated GB-inhibited diaphragm ChE to a significantly greater extent than did 2-PAM (to 62.5% of the control). TMB-4 showed the least amount of reactivation with ChE activity reaching only  $46.4 \pm 3.1\%$  of the control. Only MMB-4 and HLö7 reactivated significantly diaphragm ChE activity inhibited by GF, returning ChE activity from  $35.3 \pm 3.3\%$  to  $62.0 \pm 5.4\%$  and  $57.2 \pm 1.4\%$  of the control, respectively. They also reactivated GF-inhibited ChE significantly more than did 2-PAM (to 37.3% of the control). Carboxime, HI-6 DiCl, MMB-4, and 2-PAM were the only four oximes tested that significantly reactivated diaphragm ChE inhibited by VR, with ChE activity returning from  $48.1 \pm 4.1\%$  to  $79.0 \pm 3.8\%$ ,  $78.8 \pm 2.3\%$ ,  $77.1 \pm 6.2\%$ , and  $69.0 \pm 5.0\%$  of the control, respectively. Following VX exposure, all oximes except ICD 585 reactivated significantly inhibited diaphragm ChE. MMB-4 showed the greatest level and 2-PAM showed the least amount of reactivation, with ChE activity returning significantly from  $27.7 \pm 1.2\%$  to  $56.0 \pm 4.3\%$  and  $42.6 \pm 3.5\%$  of the control, respectively.

**Table 1**  
Cholinesterase activity in peripheral tissue and blood following exposure to nerve agents and treatments<sup>a</sup>.

Agent	Tissue	Saline	MMB-4	HLö7	Carboxime	HI-6 DMS	HI-6 DiCl	2-PAM
GB	Diaphragm	25.7 $\pm$ 2.2	90.1 $\pm$ 2.4 <sup>*,#</sup>	80.1 $\pm$ 1.4 <sup>*,#</sup>	61.4 $\pm$ 3.0 <sup>*</sup>	64.9 $\pm$ 1.9 <sup>*</sup>	62.1 $\pm$ 2.9 <sup>*</sup>	62.5 $\pm$ 3.0 <sup>*</sup>
	Heart	15.0 $\pm$ 1.5	56.2 $\pm$ 4.2 <sup>*</sup>	58.7 $\pm$ 2.8 <sup>*</sup>	56.0 $\pm$ 1.5 <sup>*</sup>	53.7 $\pm$ 0.8 <sup>*</sup>	49.4 $\pm$ 2.7 <sup>*</sup>	61.7 $\pm$ 1.5 <sup>*</sup>
	Skeletal muscle	34.2 $\pm$ 3.6	88.7 $\pm$ 3.2 <sup>*</sup>	104.8 $\pm$ 3.8 <sup>*,#</sup>	52.7 $\pm$ 2.1	69.8 $\pm$ 7.4 <sup>*</sup>	78.9 $\pm$ 4.0 <sup>*</sup>	70.2 $\pm$ 2.8 <sup>*</sup>
	RBC	8.7 $\pm$ 1.9	75.3 $\pm$ 4.2 <sup>*,#</sup>	52.1 $\pm$ 1.8 <sup>*</sup>	89.8 $\pm$ 4.3 <sup>*,#</sup>	83.1 $\pm$ 5.0 <sup>*,#</sup>	76.8 $\pm$ 2.4 <sup>*,#</sup>	52.5 $\pm$ 3.5 <sup>*</sup>
	WB	8.8 $\pm$ 1.3	83.1 $\pm$ 4.6 <sup>*,#</sup>	66.8 $\pm$ 3.9 <sup>*</sup>	74.8 $\pm$ 1.4 <sup>*</sup>	69.2 $\pm$ 4.7 <sup>*</sup>	56.9 $\pm$ 3.1 <sup>*</sup>	61.8 $\pm$ 4.0 <sup>*</sup>
GF	Diaphragm	35.3 $\pm$ 3.3	62.0 $\pm$ 5.4 <sup>*,#</sup>	57.2 $\pm$ 1.4 <sup>*,#</sup>	49.9 $\pm$ 2.5	36.6 $\pm$ 2.2	44.3 $\pm$ 5.7	37.3 $\pm$ 3.0
	Heart	34.7 $\pm$ 2.9	52.3 $\pm$ 5.6 <sup>*</sup>	43.5 $\pm$ 2.7	48.1 $\pm$ 2.2	49.0 $\pm$ 3.2	45.6 $\pm$ 3.7	41.7 $\pm$ 3.4
	Skeletal muscle	48.3 $\pm$ 4.1	75.5 $\pm$ 4.6 <sup>*,#</sup>	83.7 $\pm$ 3.8 <sup>*,#</sup>	50.5 $\pm$ 6.1	42.9 $\pm$ 4.8	58.5 $\pm$ 6.5	55.5 $\pm$ 3.4
	RBC	5.9 $\pm$ 1.1	44.8 $\pm$ 4.5 <sup>*,#</sup>	24.1 $\pm$ 1.7 <sup>*</sup>	54.3 $\pm$ 2.1 <sup>*,#</sup>	55.0 $\pm$ 5.1 <sup>*,#</sup>	44.1 $\pm$ 4.6 <sup>*,#</sup>	11.0 $\pm$ 1.5
	WB	16.6 $\pm$ 2.7	47.0 $\pm$ 2.0 <sup>*,#</sup>	45.1 $\pm$ 4.9 <sup>*,#</sup>	44.1 $\pm$ 2.7 <sup>*,#</sup>	46.7 $\pm$ 4.3 <sup>*,#</sup>	41.8 $\pm$ 3.0 <sup>*,#</sup>	20.1 $\pm$ 1.0
VR	Diaphragm	48.1 $\pm$ 4.1	77.1 $\pm$ 6.2 <sup>*</sup>	59.4 $\pm$ 5.2	79.0 $\pm$ 3.8 <sup>*</sup>	63.5 $\pm$ 4.7	78.8 $\pm$ 2.3 <sup>*</sup>	69.0 $\pm$ 5.0 <sup>*</sup>
	Heart	54.9 $\pm$ 2.0	97.8 $\pm$ 6.0 <sup>*,#</sup>	74.6 $\pm$ 4.8 <sup>*</sup>	96.9 $\pm$ 4.0 <sup>*,#</sup>	82.0 $\pm$ 4.8 <sup>*</sup>	69.3 $\pm$ 2.7	68.9 $\pm$ 2.5
	Skeletal muscle	60.9 $\pm$ 4.0	81.1 $\pm$ 6.0 <sup>*,#</sup>	63.4 $\pm$ 5.2	77.6 $\pm$ 4.4	79.7 $\pm$ 4.6	95.1 $\pm$ 3.0 <sup>*</sup>	92.7 $\pm$ 3.3 <sup>*</sup>
	RBC	6.6 $\pm$ 1.2	81.2 $\pm$ 9.8 <sup>*,#</sup>	58.4 $\pm$ 3.2 <sup>*,#</sup>	91.9 $\pm$ 2.8 <sup>*,#</sup>	45.6 $\pm$ 3.3 <sup>*,#</sup>	39.8 $\pm$ 2.4 <sup>*,#</sup>	6.6 $\pm$ 2.0
	WB	30.8 $\pm$ 3.6	80.5 $\pm$ 2.2 <sup>*,#</sup>	89.1 $\pm$ 5.4 <sup>*,#</sup>	89.3 $\pm$ 0.8 <sup>*,#</sup>	73.6 $\pm$ 9.5 <sup>*,#</sup>	73.2 $\pm$ 4.1 <sup>*,#</sup>	50.2 $\pm$ 4.7
VX	Diaphragm	27.7 $\pm$ 1.2	56.0 $\pm$ 4.3 <sup>*</sup>	48.0 $\pm$ 3.5 <sup>*</sup>	56.2 $\pm$ 1.1 <sup>*</sup>	50.3 $\pm$ 2.1 <sup>*</sup>	44.3 $\pm$ 2.8 <sup>*</sup>	42.6 $\pm$ 3.5 <sup>*</sup>
	Heart	23.6 $\pm$ 1.1	54.6 $\pm$ 3.9 <sup>*</sup>	63.0 $\pm$ 3.6 <sup>*,#</sup>	62.5 $\pm$ 2.1 <sup>*,#</sup>	44.2 $\pm$ 1.3 <sup>*</sup>	38.9 $\pm$ 1.4 <sup>*</sup>	44.1 $\pm$ 3.8 <sup>*</sup>
	Skeletal muscle	32.1 $\pm$ 2.4	56.1 $\pm$ 3.4 <sup>*,#</sup>	56.7 $\pm$ 3.7 <sup>*</sup>	49.1 $\pm$ 1.8 <sup>*</sup>	51.9 $\pm$ 2.3 <sup>*</sup>	46.3 $\pm$ 3.0 <sup>*</sup>	46.6 $\pm$ 2.2 <sup>*</sup>
	RBC	6.3 $\pm$ 1.0	83.9 $\pm$ 2.1 <sup>*,#</sup>	84.4 $\pm$ 2.6 <sup>*,#</sup>	84.7 $\pm$ 3.4 <sup>*,#</sup>	56.8 $\pm$ 2.3 <sup>*,#</sup>	44.3 $\pm$ 2.4 <sup>*</sup>	37.7 $\pm$ 4.7 <sup>*</sup>
	WB	26.8 $\pm$ 2.1	82.7 $\pm$ 1.9 <sup>*,#</sup>	66.4 $\pm$ 4.2 <sup>*,#</sup>	78.2 $\pm$ 2.1 <sup>*,#</sup>	56.3 $\pm$ 1.6 <sup>*</sup>	46.2 $\pm$ 1.7 <sup>*</sup>	43.5 $\pm$ 3.2 <sup>*</sup>

<sup>a</sup> Effects of nerve agent and saline or oxime treatments on peripheral tissue ChE activity in the guinea pig. Saline or oxime compounds was given intramuscularly 5 min after a  $1.0 \times LD_{50}$  subcutaneous dose of GB, GF, VR or VX. Samples were collected at 60 min after nerve agent. ChE activity in diaphragm, heart, and skeletal muscle was expressed as percent of control ChE activity for each tissue. ChE activity in RBC and WB was expressed as percent of individual baseline activity that was obtained 1–3 days prior to experiment. Total ChE activity was expressed as mean  $\pm$  SEM (% of control group) with 6–8 animals per group.

<sup>\*</sup> Significantly different from saline treatment,  $p < 0.05$ .

<sup>#</sup> Significantly different from 2-PAM treatment,  $p < 0.05$ .



The non-oximes (SAD128 and ICD4157) did not modify the ChE activities inhibited by any nerve agent in the diaphragm, with one exception. The ChE activity showed an elevation from  $25.7 \pm 2.2\%$  to  $39.8 \pm 5.0\%$  of the control in animals exposed to GB and treated with SAD128.

### 3.3.2. Heart tissue

All oximes were capable of significantly reactivating ChE activity inhibited by GB in the heart. 2-PAM showed the greatest and TMB-4 the least amount of reactivation, with ChE returning from  $15.0 \pm 1.5\%$  to  $61.7 \pm 1.5\%$  and  $32.9 \pm 0.9\%$  of the control, respectively. Following GF exposure, only MMB-4 reactivated significantly inhibited heart ChE with activity returning from  $34.7 \pm 2.9\%$  to  $52.3 \pm 5.6\%$  of the control. MMB-4, carboxime, HLö7, HI-6 DMS, and ICD692 were capable of significantly reactivating heart ChE inhibited by VR. MMB-4 and carboxime exhibited the highest levels of reactivation following VR exposure, returning ChE activity from  $54.9 \pm 2.0\%$  to  $97.8 \pm 6.0\%$  and  $96.9 \pm 4.0\%$  of the control, respectively. They reactivated VR-inhibited ChE significantly more than did 2-PAM (to  $68.9 \pm 2.5\%$  of the control). HLö7 showed the least significant amount of reactivation in VR-inhibited heart ChE, where activity returned to  $74.6 \pm 4.8\%$  of the control value. Only ICD585 was not capable of reactivating heart ChE activities inhibited by VX. HLö7 showed the greatest and HI-6 DiCl the least significant amount of reactivation with VX-inhibited ChE returning from  $23.6 \pm 1.1\%$  to  $63.0 \pm 5.6\%$  and  $38.9 \pm 1.4\%$  of the control, respectively. HLö7 and carboxime (to  $62.5\%$  of the control) reactivated heart ChE significantly more than did 2-PAM (to  $44.1\%$  of the control).

The non-oximes (SAD128 and ICD4157) did not modify the ChE activities inhibited by any nerve agent in the heart.

### 3.3.3. Skeletal muscle tissue

All oximes studied, with the exception of carboxime and TMB-4, were capable of significantly reactivating skeletal muscle ChE activity inhibited by GB. HLö7 showed the greatest level of reactivation with ChE activity returning from  $34.2 \pm 3.6\%$  to  $104.8 \pm 3.8\%$  of the control. ICD3805 and MMB-4 also displayed excellent levels of reactivation, with ChE reaching  $89.7 \pm 3.6\%$  and  $88.7 \pm 3.2\%$  of the control, respectively. Only MMB-4 and HLö7 reactivated significantly skeletal muscle ChE activity inhibited by GF, with the ChE activity returning from  $48.3 \pm 4.1\%$  to  $75.5 \pm 4.6\%$  and  $83.7 \pm 3.8\%$  of the control, respectively. MMB-4, HI-6 DiCl and 2-PAM were the only three oximes tested that significantly reactivated skeletal muscle ChE activity inhibited by VR. HI-6 DiCl showed greater reactivation than did 2-PAM and MMB-4, returning ChE activity from  $60.9 \pm 4.0\%$  to  $95.1 \pm 3.0\%$ ,  $92.7 \pm 3.3\%$ , and  $81.1 \pm 6.0\%$  of the control, respectively. All oximes except ICD585 significantly reactivated skeletal muscle ChE activity inhibited by VX. HLö7 showed the greatest and HI-6 DiCl showed the least amount of reactivation with ChE activity returning from  $32.1 \pm 2.4\%$  to  $56.7 \pm 3.7\%$  and  $46.3 \pm 3.0\%$  of the control, respectively. The non-oximes (SAD128 and ICD4157) did not modify the ChE activities inhibited by any nerve agent in the skeletal muscle, with one exception. The ChE activities showed an elevation from  $34.2 \pm 3.6\%$  to  $57.4 \pm 8.2\%$  of the control in animals exposed to GB and treated with SAD128.

### 3.4. ChE activity in blood (Table 1)

The basal ChE activities in RBC and WB are  $2.2 \pm 0.06$  and  $2.5 \pm 0.06 \mu\text{mol}/(\text{ml min})$ , respectively. The ChE activities in RBC were markedly inhibited 60 min following GB, GF, VR, and VX to  $8.7 \pm 1.9\%$ ,  $5.9 \pm 1.1\%$ ,  $6.6 \pm 1.2\%$ , and  $6.3 \pm 1.0\%$  of control value, respectively. Thus, all four nerve agents produced a similar degree of RBC ChE inhibition. In the WB, however, GB, GF, VR and VX reduced enzyme activity to  $8.8 \pm 1.3\%$ ,  $16.6 \pm 2.7\%$ ,  $30.8 \pm 3.6\%$ , and

$26.8 \pm 2.1\%$  of control value, respectively. Both GB and GF produced significantly greater ChE inhibition than did VR and VX. The latter two nerve agents showed a similar degree of ChE inhibition.

The non-oximes (SAD128 and ICD4157) did not significantly modify the ChE activities inhibited by any nerve agent in any blood samples.

### 3.4.1. Red blood cell (RBC)

All nine oximes studied were capable of significantly reactivating RBC ChE activity inhibited by GB. ICD692 (90.2% of control), carboxime (89.8% of control), HI-6 DMS (83% of control), HI-6 DiCl (77% of control), ICD585 (76% of control), and MMB-4 (75% of control) were capable of reactivating GB-inhibited RBC ChE activity significantly more than 2-PAM (53% of control). TMB-4 showed the least amount of reactivation with enzyme activity returning from  $8.7 \pm 1.9\%$  to  $29.0 \pm 1.3\%$  of the control. All oximes except 2-PAM and TMB-4 significantly reactivated RBC ChE activity inhibited by GF. HI-6 DMS and carboxime showed the highest levels of reactivation, returning ChE activity from  $5.9 \pm 1.1\%$  to  $55.0 \pm 5.1\%$  and  $54.3 \pm 2.1\%$  of the control, respectively. HLö7 showed the least amount of reactivation with ChE activity returning to  $24.1 \pm 1.7\%$  of the control. All oximes except 2-PAM and ICD585 were capable of significantly reactivating RBC ChE activity inhibited by VR. Carboxime and MMB-4 showed the highest levels of reactivation with ChE returning from  $6.6 \pm 1.2\%$  to  $91.9 \pm 2.8\%$  and  $81.2 \pm 9.8\%$  of the control, respectively. TMB-4 showed the least amount of reactivation among these oximes with ChE activity reaching only  $27.5 \pm 4.7\%$  of the control. All oximes significantly reactivated RBC ChE activity inhibited by VX. MMB-4, carboxime, HLö7, and ICD3805 showed excellent levels of reactivation with ChE activity returning from  $6.3 \pm 1.0\%$  to  $83.9 \pm 2.1\%$ ,  $84.7 \pm 3.4\%$ ,  $84.4 \pm 2.6\%$ , and  $83.9 \pm 2.7\%$  of the control, respectively. HI-6 DMS showed the least amount of reactivation, with ChE activity returning to  $56.8 \pm 2.3\%$  of the control.

### 3.4.2. Whole blood (WB)

All oximes studied significantly reactivated ChE activity inhibited by GB. MMB-4 showed the greatest and TMB-4 showed the least amount of reactivation with WB ChE activity returning from  $8.8 \pm 1.3\%$  to  $83.1 \pm 4.6\%$  and  $50.5 \pm 2.7\%$  of the control, respectively. MMB-4 reactivated GB-inhibited WB ChE activity significantly more than did 2-PAM (to  $61.8\%$  of the control). All oximes except 2-PAM, ICD585, and TMB-4 were capable of significantly reactivating WB ChE activity inhibited by GF. MMB-4 showed the greatest and ICD692 showed the least amount of reactivation with ChE activity returning from  $16.6 \pm 2.7\%$  to  $47.0 \pm 2.0\%$  and  $36.7 \pm 2.4\%$  of the control, respectively. All oximes except 2-PAM and ICD585 significantly reactivated WB ChE activity inhibited by VR. Carboxime and HLö7 showed the highest levels of reactivation such that ChE activity returned from  $30.8 \pm 3.6\%$  to  $89.3 \pm 0.8\%$  and  $89.1 \pm 5.4\%$  of the control, respectively. ICD692 showed the least amount of reactivation among these oximes with ChE activity returning to  $63.7 \pm 4.7\%$  of the control.

All oximes significantly reactivated WB ChE activity inhibited by VX. MMB-4 and carboxime showed the highest levels of reactivation with ChE activity returning from  $26.8 \pm 2.1\%$  to  $82.7 \pm 1.9\%$  and  $78.2 \pm 2.1\%$  of the control, respectively. HI-6 DiCl showed the least amount of reactivation with a return in ChE activity to  $46.2 \pm 1.7\%$  of the control.

### 3.5. Overall assessment of oxime reactivating capacity

Table 2A and B summarized the statistically significant ChE reactivation by oxime treatments in the peripheral tissues and the blood components, respectively. It is striking to notice that MMB-4 is the only oxime capable of significantly reactivating ChE inhibited by

**Table 2**Summary of the significant ChE reactivation by oxime treatments in peripheral tissues and blood components following exposure to GB, GF, VR, and VX<sup>a</sup>.

A. Peripheral tissues (diaphragm, heart, skeletal muscle)										
	MMB-4	HLö7	Carboxime	HI-6 DMS	HI-6 DiCl	2-PAM	ICD585	ICD692	ICD3805	TMB-4
GB	+++	+++	++	+++	+++	+++	+++	+++	+++	++
GF	+++	++	—	—	—	—	—	—	—	—
VR	+++	+	++	+	++	++	—	+	—	—
VX	+++	+++	+++	+++	+++	+++	—	+++	+++	+++
B. Blood (red blood cells and whole blood)										
	MMB-4	HLö7	Carboxime	HI-6 DMS	HI-6 DiCl	2-PAM	ICD585	ICD692	ICD3805	TMB-4
GB	++	++	++	++	++	++	++	++	++	++
GF	++	++	++	++	++	—	+	++	++	—
VR	++	++	++	++	++	—	—	++	++	++
VX	++	++	++	++	++	++	++	++	++	++

<sup>a</sup> For each of the peripheral tissues (A) or blood components (B), an oxime treatment that significantly reactivated a nerve agent-inhibited ChE activity was assigned a “+” sign. When an oxime treatment did not significantly reactivate a nerve agent-inhibited ChE in any tissue, it was assigned a “—” sign.

all four nerve agents in all three peripheral tissues. HLö7 was also capable of reactivating ChE inhibited by these four nerve agents, but with a weaker action toward VR- and GF-inhibited tissue ChE activity. The reactivating potency of carboxime, HI-6 DMS, HI-6 DiCl, 2-PAM and ICD692 appears to be equal. They all lacked the ability to reactivate GF-inhibited ChE in the peripheral tissues. The least effective oxime, ICD 585 was not able to reactivate peripheral tissue ChE activity inhibited by GF, VR or VX. The relative ChE reactivating potency of these oxime compounds against the four nerve agents was GB > VX > VR > GF. The DiCl and DMS salts of HI-6 exhibited similar potency against GB, GF, VR and VX (Table 1).

#### 4. Discussion

As was reported earlier OP nerve agents produced tissue compartment specificity in their ability to inhibit ChE activity [20]; the oximes studied here (i.e., 2-PAM, HI-6, HLö7, MMB-4, TMB-4, carboxime, ICD585, ICD692, and ICD3805) also exhibited differential potency in reactivating nerve agent-inhibited tissue ChE activity. When examined carefully the results obtained from the present study showed that MMB-4 was the only oxime (among 9 oxime compounds tested) capable of reactivating ChE inhibited by all four nerve agents in all three peripheral tissues and the blood components. Additionally, in several cases, ChE activities in animals treated with MMB-4 reached control values: in diaphragm and skeletal muscle after GB and in heart after VR exposures. HLö7 was second in its overall reactivating capacity. In RBC and WB it reactivated ChE inhibited by all four nerve agents. Skeletal muscle ChE activities in animals treated with HLö7 after GB exposure returned to control value. Although HLö7 was capable of reactivating ChE in all three peripheral tissues inhibited by GB and VX, it lacked the ability to reactivate ChE inhibited in the diaphragm and skeletal muscle by VR and ChE inhibited in the heart by GF. Carboxime reactivated ChE in RBC and WB inhibited by all four nerve agents and was capable of reactivating VX-inhibited ChE in all three and GB-inhibited ChE in two peripheral tissues. It returned the ChE activity in the heart to the control value after VR exposure. However, carboxime was not able to reactivate GF-inhibited ChE in diaphragm, heart, and skeletal muscle, and VR-inhibited ChE in skeletal muscle. HI-6 showed a complete nerve agent preference. It was capable of reactivating GB-, VR- and VX-inhibited ChE, but not GF-inhibited ChE, in blood or tissues. There was no difference in ChE reactivating capacity between the two salts of HI-6 following exposure to the four nerve agents studied. Similar results were reported by laboratory in an *in vitro* study [16]. 2-PAM was an excellent reactivator of ChE activity inhibited by GB and VX in blood, and in all three peripheral tissues. It was not able to reactivate GF-inhibited ChE

in blood and any peripheral tissue tested, or VR-inhibited ChE in blood and the heart. On the other hand, although the other three ICD compounds (ICD585, ICD692, and ICD3805) were, in general, able to reactivate ChE in the blood inhibited by all four nerve agents, these compounds lacked the capability to do so on GF- and VR-inhibited ChE in any peripheral tissues. Overall, ICD 585 was not able to reactivate GF-, VR-, and VX-inhibited ChE, but was able to only reactivate GB-inhibited ChE in all three peripheral tissues.

The present results also showed that these oximes were able to reactive ChE inhibited by four different nerve agents in peripheral tissues to different degrees (Table 2). The rank order of susceptibility for *in vivo* ChE reactivation by oxime treatments in peripheral tissues was GB > VX > VR > GF.

It is interesting to note that the capability of an oxime treatment to reactivate ChE in the blood and the peripheral tissue compartments was markedly different. It was understandable that the oxime compound entered the blood first before reacting in the tissue compartment, and thus, it had a better chance to act on the ChE inhibited in the blood (i.e., proximity effect). Therefore, it was observed that most of the oximes were capable of reactivating nerve agent-inhibited ChE in the blood to a greater degree than in the peripheral tissues. For example, with the exception of 2-PAM and TMB-4, all oximes tested displayed significant ChE reactivation in the blood components, while in the peripheral tissue only MMB-4 and HLö7 were capable of doing so. Furthermore, the majority of the oximes studied were capable of reactivating ChE inhibited by the four nerve agents in the blood components significantly better than was 2-PAM. In the peripheral tissue, however, only MMB-4, HLö7, or carboxime displayed significantly better ChE reactivating capacity than did 2-PAM, but only in a few tissues. There was no relation between blood and peripheral tissues in the reactivating efficacy of oxime treatments. Therefore, a higher blood ChE activity following oxime treatment in nerve agent poisoning could not be used as a direct indicator of elevated ChE activity in a peripheral tissue (diaphragm, heart, or skeletal muscle).

It is also interesting to pay attention to an issue brought up by a few *in vitro* studies in recent years [23–25] using RBC ghosts as AChE sources, which have demonstrated that oxime reactivation of guinea pig AChE *in vitro* is significantly slower than that of human or monkey AChEs, especially in the reactivation of GF-, soman-, or VR-inhibited enzyme by HI-6 and HLö7. However, these *in vitro* experiments [24,25] were conducted with a pH of 8.0 at 25 °C, which is not the optimal physiological condition in living animals. Therefore, these results are not easily reconcilable with our *in vivo* guinea pig data. Even the study of Worek et al. [23] that was performed at a pH of 7.4 at 37 °C, whose *in vitro* kinetic data obtained from guinea pig RBC ghosts were markedly different

from current *in vivo* ChE reactivation data obtained from guinea pig RBC. For example, *in vitro* reactivation kinetic data indicated that GB-inhibited ghost AChE would respond less to HL07, HI-6 and 2-PAM, in particular to HI-6, while the *in vivo* results showed that HI-6 was the most effective RBC AChE reactivator when compared with HL07 and 2-PAM (Table 1). Additionally, as discussed earlier a higher blood ChE activity following oxime treatment in nerve agent poisoning could not be used to predict ChE activity in a peripheral tissue (diaphragm, heart, or skeletal muscle). Whether the AChE reactivation responses in erythrocyte ghosts could be used to predict human tissue responses are not yet settled. Therefore, more studies using other *in vivo* animal models and *in vitro* tissue models will be necessary to resolve this issue.

In this study, two structurally similar non-oxime compounds, SAD128 and ICD4157, were used as negative controls for ChE reactivation analysis. There were some differences between the actions of these two compounds. ICD4157 acted just like a saline control treatment; it neither reactivated nerve agent-inhibited ChE in blood and peripheral tissues, nor affected either GB- or GF-induced mortality within 60 min after agent exposure when compared with nerve agent/saline treatment. In contrast, SAD128 treatment prevented mortality when compared with the nerve agent/saline treatment (0% and 11% for GB and GF exposure, respectively). It also had significantly elevated ChE activity in diaphragm and in skeletal muscle of GB-exposed animals when compared with the GB/saline treatment group. This finding confirmed the observations made for SAD128 by Clement and Erhardt [26] reported in an *in vitro* study that SAD128 treatment 5 min after exposure to soman resulted in significantly greater total ChE activity in rat diaphragm tissue. There have been many speculations on the beneficial effects of SAD128, for example, shielding of ChE from inhibition by soman [27,28], blockage of the muscarinic receptors [29], or blockage of the nicotinic receptor ion channel [30]. However, we observed reactivation only in the case of GB-inhibited tissue ChE. The reason for this is not clear.

In summary, the present results showed that the OP nerve agents GB, GF, VR, and VX caused different degrees of ChE inhibition in various tissue compartments. The oximes also showed differential potency in reactivating nerve agent-inhibited ChE in various peripheral tissues and exhibited differential ChE reactivating specificity for nerve agents. Oxime induced reactivation of nerve agent-inhibited ChE in the brain or spinal cord was not clearly demonstrated. ChE inhibited by GB was most susceptible and that inhibited by GF was the least susceptible to oxime reactivation. A higher blood ChE activity following oxime treatment in nerve agent poisoning could not be used as a direct indicator of elevated ChE activity in a peripheral tissue. There was no difference in the ChE reactivating potency of the DiCl and DMS salts of HI-6 in any blood or peripheral tissue compartment. Among all nine oxime compounds tested, MMB-4 was the only oxime capable of reactivating ChE inhibited by all four nerve agents in all three peripheral tissues of the guinea pig. MMB-4, thus, appears to be the broadest spectrum *in vivo* reactivator for GB, GF, VX and VR intoxication. The relationship between the ChE reactivation observed with these oximes in peripheral tissues and the survival following exposure to these nerve agents is currently under investigation.

## Conflict of interest

The authors declare that there are no conflicts of interests.

## Acknowledgments

The authors recognized the excellent technical assistance of Laura Gildner, Megan Lyman, Emily Mason, Maura Pannell, and

Tami Rowland. This research was supported by the Defense Threat Reduction Agency—Joint Service and Technology Office, Medical Science and Technology Division.

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